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Calcium A Key Player In Early Neural Development And Migration: TRPCs and VGCCs

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Academic Dissertation

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Abstract

Signaling pathways linked to changes in the intracellular cytosolic concentrations of free calcium are involved in a plethora of cellular activities ranging from cell division, differentiation and migration, integration of neural circuits, as well as programmed neuronal cell death. Channels that permit the entrance of calcium into neuronal cells have gathered considerable interest due to their potential of being significant therapeutic targets not least to mention a means by which to answer key neurophysiological questions.

This thesis investigates two distinct calcium influx pathways: one mediated by the transient receptor potential channel family which can be either directly activated or indirectly via second messengers and the second pathway involving voltage gated calcium channels.

Using a neuronal cell line model (human neuroblastoma IMR-32) as well as the neural progenitor stem cell neurosphere assay with molecular biology techniques, immunocytochemistry, calcium imaging, electrophysiology and time-lapsed imaging the thesis culminates to provide novel insights into the interplay between these channel proteins and the differentiation and migration of neuronal cells. Furthermore it provides new information on the molecular mechanisms involved in the interaction between developing neurons and radial glial cells, whereby the migrational behavior of neuronal cells is influenced by the calcium influx pathways mediated by glutamate receptors in radial glial cells.

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Lauri Louhivuori

List of original publications:

This thesis is based on the following publications that will be referred to in the text by their Roman numerals (I-IV):

I. **Louhivuori LM**, Bart G, Larsson KP, Louhivuori V, Näsman J, Nordström T, Koivisto AP, Akerman KE. Differentiation dependent expression of TRPA1 and TRPM8 channels in IMR-32 human neuroblastoma cells. *J Cell Physiol*. 2009 Oct;221(1):67-74.

II. **Louhivuori LM**, Jansson L, Nordström T, Bart G, Näsman J, Akerman KE. Selective interference with TRPC3/6 channels disrupts OX1 receptor signalling via NCX and reveals a distinct calcium influx pathway. *Cell Calcium*. 2010 Aug-Sep;48(2-3):114-23.

III. **Louhivuori LM** Jansson LC, Turunen P, Jäntti MH, Nordström T, Louhivuori V, Akerman KE. TRPC channels and their role in modulating radial glial-neuronal interaction: a signaling pathway involving mGluR5. *Stem Cells Dev.* 2015 Mar 15;24(6):701-13

IV. **Louhivuori LM**, Louhivuori V, Wigren HK, Hakala E, Jansson LC, Nordström T, Castrén ML, Akerman KE. Role of low voltage activated calcium channels in neuritogenesis and active migration of embryonic neural progenitor cells. *Stem Cells Dev.* 2013 Apr 15;22(8):1206-19.

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Abbreviations

$[Ca^{2+}]_i$	intracellular free calcium concentration
$[Ca^{2+}]_e$	extracellular free calcium concentration
$[Na^{+}]_i$	intracelluar sodium concentration
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
CRAC	calcium release activated calcium channel
cGMP	cylic guanosine-5'-monophosphate
CaCA	Ca^{2+}/cation antiporter genes
CNS	central nervous system
DAG	diacylglycerol
DAGK	diacylgylcerol kinase
DAGL	diacylgylcerol lipase
DHPG	3,5-dihydroxyphenylglycine
EBSS	Earl's balanced salt solution
EGF	epidermal growth factor
ER	endoplasimc reticulum
FGF	fibroblast growth factor
FVB	Friends virus B
GABA	γ-aminobutyric acid
GPCR	guanine protein coupled receptor(s)
GDP	guanosine-5'-diphosphate
GLAST	glutamate/aspartate transporter
GTP	guanosine-5'-triphosphate
HBM	hepes buffered media
HBSS	Hank's buffered salt solution
HEK293	human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVA	high voltage activated
IP_3	inositol-1,4,5-trisphosphate
IP_3R	inositol-1,4,5-trisphosphate receptor
KB-R7943	2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate
KO	knock-out
LVA	low voltage activated
MAP-2	microtubule associated protein-2

mGluR	metabotropic glutmate receptor
mRNA	messenger ribonucleic acid
NCX	sodium calcium exchanger
NMDA	N-methyl-D-aspartate
NMDG	N-methyl-D-glucamine
NPC	neural progenitor cell
NSC	neuronal stem cell
Ox1R	orexin-1 receptor
Ox2R	orexin-2 receptor
OxA	orexin-A
OxB	orexin-B
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PKC	protein kinase C
PI	phosphatidylinositol
PIP_2	phosphatidylinositol-4,5-bisphosphate
ROC	receptor operated channel
RPM	rotations per minute
RT-PCR	real time polymerase chain reaction
SOC	store operated channel
SLC	solute carrier
STIM	stromal interacting molecule
TRP	transient receptor potential
TRPC	canonical transient receptor potential
TRPA1	transient receptor potential anykrin 1 channel
TRPM8	transient receptor potential melanin 8 channel
TRPML	transient receptor potential mucolipin
TRPP	transient receptor potential polycystine
TRPV	transient receptor potential vanniloid
TTX	tetrodotoxin
VGCC	voltage gated calcium channels

1. Introduction

The largest structure of the human brain is the cerebral cortex. It is without a doubt one of the crowning achievements of evolution and at the core of what makes us quintessentially human. The cyto-architecture of the mature cortex consists of six discernable layers composed of a network of billions of excitatory neurons and inhibitory interneurons. Its development and lamination requires the rigorous control of cellular and molecular events whose temporal and sequential developmental profile is precisely orchestrated by both intrinsic and extrinsic factors.

One such feature is the migration and differentiation of the cortical precursor cells which emanate from the neural stem cells known as the radial glial cells. This process of production, allocation, migration, and settling of neurons into their correct positions within the cortical sheet is known as corticogenesis. The consequence of failure or delay in neuronal migration causes a variety of disorders including lissencephaly, mental retardation, autism, and schizophrenia (reviewed in (Guerrini and Dobyns, 2014; Marin, 2012).

The molecular mechanisms responsible for enacting the effect of extracelluar guiding ques, let alone the intrinsic locomotive driving force and the protein machinery involved in neuronal motility, are not yet fully understood. Accumulating evidence indicates that changes in the cytosolic calcium concentrations plays an important and diverse role in the migratory process, including responses to cues and regulation of molecular motors involved in motility. Disorders in the pathways leading to changes in intracellular calcium have profound effects on both the development and maturation of neurons (Leclerc et al., 2011).

As such the work presented in this thesis addresses the role membrane channel proteins permeable to calcium play in the differentiation of neuronal cells as they mature and migrate in an in vitro setting that allows the careful study of individual cells in detail. The different projects follow a natural progression of understanding that at first were derived from studies on a model neuronal cell line and which subsequently led to questions and answered using embryonic progenitor stem cells. The thesis will begin with a brief overview of the historical background of cortical neurogenesis, followed by a review of calcium signaling with particular emphasis on calcium influx pathways.

2. Review of the literature

2.1 Cortical neurogenesis: A Historical Perspective

A fundamental feature of cerebral cortical development is the division of cells into functionally distinct areas which are composed of radial columns of neurons that share similar biophysical properties interconnected via a multitude of profuse axons and which receive and process peripheral input. Most of the cellular events described to be involved in corticoneurogensis date back to the late 19th century as well as the first identifications of the radial glial cell populations (for those interested in a detailed historical account which goes beyond the scope of this thesis, the following review is suggested (Bentivoglio and Mazzarello, 1999)). Groundbreaking to say the least were the human fetal histology work of done by Kölliker and His (His, 1904; Kölliker, 1879; Kölliker, 1882; Kölliker, 1896) that identified among many other things the presence of cells with long radial morphology (i.e. arranged like rays or having spokes protruding like brushes). These dividing cells were found to be positioned along the lumen of the cerebral ventricles and furthermore that they were found to be undetectable in the cortical regions developing under the pial surface (His, 1889). Golgi using the then revolutionary silver impregnation techniques was the first to systematically describe the radially aligned cells to be of apparent glial lineage and hence distinct from (neural)epitheial cells (Golgi, 1885). A few years later the Italian physiologist Magini (Magini, 1888) undertook further investigations and focused on the particular swellings that he observed on the radial glia. Utilizing the Golgi method and staining with hematoxylin he was able to identify nuclei in those varicose structures and hypothesized that they could be nerve cell precursors. Remarkably he went on to suggest that the radial glia could function as a migrational scaffold for neurons (Bentivoglio and Mazzarello, 1999). At around the same time Ramon y Cajal and von Lenhossék attempting to decipher whether the elongated radially morphed cells were neuroblasts or not, demonstrated that radial glia differentiated into structures that were astrocyte-like and thus glial in nature (reviewed in (Garcia-Marin et al., 2007)). Remarkably these anatomical studies done over a century ago still resonate and hold true to a certain degree till this day.

As with most scientific endeavors, the dawning of new techniques heralds new insights into past discoveries. It was not until the early 1970s with the arrival of electron microscope technique that the painstaking work performed by Rakic on midgestational human and monkey cortical slices demonstrated that the majority of neurons migrating out of the ventricular zone were in close contact with radial glia fibers as they journeyed to their final destination. This provided additional support to the idea that radial glia

cells acted as a scaffold for neuronal migration (Rakic, 1972; Sidman and Rakic, 1973) reviewed in (Sild and Ruthazer, 2011). Studies using modern techniques in immunostaining, and time-lapse imaging on midgestational rat embroys validated the migration of neurons along radial glia fibers (Noctor et al., 2001). In addition, the same study went on to reveal that the radial glial cells were actually the precursors for the diverse population of cortical cells including both neurons and glia (Noctor et al., 2001; Tamamaki et al., 2001).

2.2 Migration of cortical neurons

The excitatory glutamatergic projection neurons, which project to numerous cortical and subcortical regions, and the inhibitory GABAergic local circuit interneurons, which as the name suggests make local connections within the cortex, are the two main classes of cortical neurons (reviewed in (Molyneaux et al., 2007). They are both found throughout the six layered cortical sheets; however GABAergic interneurons make up for only one fifth of the total amount of neurons in the cortex (Hendry et al., 1987).

The source of glutamergic neurons stem from the neuroepithelial cells which line the walls of the lateral cerebral ventricles, whereas the GABAergic interneurons originate from medial-, lateral-, and caudal ganglionic eminences of the ventral telencephalon (reviewed in (Kriegstein and Alvarez-Buylla, 2009)). Once the newborn postmitotic glutamergic neurons exit the proliferative zone, they migrate along pial process of the radial glial cells through a process termed radial migration (Haydar et al., 1999; Kawaguchi et al., 2001; Noctor et al., 2001; Rakic, 1974). This mode of migration is characterized by phases of saltatory motion followed by phases with sporadic pauses. The radial unit hypothesis encompasses a theoretical framework whereby i) the final horizontal cortical positions of the corticoneurons is determined by the relative position of the precursor cells in the subventricular zone, and ii) the vertical position is a reflection of the time of their origin (Rakic et al., 2009).

On the other hand, GABAergic interneurons migrate to the cortex by a form of locomotion known as tangential migration (Anderson et al., 1997; de Carlos et al., 1996; Jimenez et al., 2002; Lavdas et al., 1999; Lois and Alvarez-Buylla, 1994; Nery et al., 2002; Polleux et al., 2002; Tamamaki et al., 1997; Taniguchi et al., 2011; Wichterle et al., 1999; Yozu et al., 2005) which unlike radial migration, migrate on or in close association with each other either independently of any specific substrate or along axonal fibers (Martin et al., 2003).

It comes as no surprise then that neuronal motility is one of the fundamental features that underlies the development of the nervous system and is essential for normal neocortical function.

Despite these hallmark studies the molecular mechanisms responsible for carrying out the effect of extracelluar guiding ques are less well investigated. Accumulating evidence indicates that changes in the cytosolic calcium concentrations plays an important and diverse role in the migratory process, including responses to cues and regulation of molecular motors involved in motility. In this context, recent evidence suggests that nonselective cation channels of the canonical transient receptor potential (TRPC) channels family mediate responses to chemoattractant and repellant factors.

2.3 Principles of calcium signaling

Ask a layman's opinion on calcium and they will answer "milk" and "and strengthens my bones and teeth". Yes, calcium is the fifth most abundant element in the Earth's crust (with only oxygen, silicon, aluminum and iron having more mass) however for those working in the field of cell signaling, most if not all will most evidently agree that ionized calcium is by far the most widely used universal second messenger in organisms inhabiting this planet. The reason is quite simple in essence. Calcium has a highly eager nature of interacting with biological molecules and this is a result of its chemical properties (high affinity for the most frequent motif in amino acids: carboxylate oxygen, its flexible coordination chemistry, rapid binding kinetics to name a few). Thus already most likely during the formation of the first cells in the primordial volcanic soup millions of years ago, cells evolved highly specialized membrane and cytosolic proteins whose primary functions was to limit the amount of unbound and free ionized calcium within its enclosed membrane environment maintaining in rest conditions a concentration of 100nM $[Ca^{2+}]_i$ compared to the near 10 000 to 20 000 times greater concentration outside the cell (1-2mM).

The factors involved in bringing about this steep Ca^{2+} concentration are i) the activity of a number of evolutionarily highly conserved calcium transporters and channels that permit the movement of Ca^{2+} ions either down their concentration gradient or against it and ii) the presence of Ca^{2+} storage compartments within the cell (Verkhratsky, 2006).

A byproduct of this strict control in Ca^{2+} management is that the cell now has a good signaling molecule due to the very low signal-to-noise ratio. Thus, as time passed cells evolved and added more complicated forms of signaling via receptors that utilized the Ca^{2+} signaling machinery to mediate their responses. It should come as no surprise then

that Ca^{2+} is involved in nearly every cell behavior ranging from migration, to development of neural cells, the integration of neural circuits, as well as programmed cell death (Wes et al., 1995).

2.3.1 Calcium influx pathways

The influx of Ca^{2+} from the extracellular milieu into the cells is controlled by various membrane proteins that are permeable to Ca^{2+} ions, including voltage gated calcium channels which open in response to changes in the voltage across cell membranes, receptor operated channels that open as a result second messenger cascades initiated by the binding of an extracelluar ligand to a receptor, and store operated channels which open after the discharge of Ca^{2+} from internal stores.

2.3.2 Voltage gated calcium channels

In excitable cells the voltage gated calcium channels (VGCC) undertake a unique role by rapidly transforming membrane events into cytosolic Ca^{2+} signals. The influx of Ca^{2+} via VGCCs is modulated by changes in membrane potential which are coupled to numerous intracellular mechanisms that are dependent on Ca^{2+} such as neurotransmitter release, gene expression, and differentiation.

The VGCC can be divided into two groups based on their threshold for activation 1) low-voltage activated (LVA) calcium channels which, as the name suggests, are activated by small changes in membrane potential and 2) high-voltage activated (HVA) VGCCs that require a relatively high step in membrane voltage to open (Hagiwara et al., 1975; Llinas and Yarom, 1981)

One of the first widely known members of the HVA channel family is found extensively in all muscle tissue (cardiac, smooth and skeletal) and in neurons. The early electrophysiological studies performed on these channels recorded currents with large amplitudes in single channel conductance as well as slow kinetics and hence they were named L-type calcium channels (L for large and long lasting). LVA channels due to their small single channel conductance amplitudes and fast decaying currents were coined T-type calcium channels (T for tiny and transient) (Hagiwara et al., 1975).

Experiments performed in the 1980s on neuronal cells revealed novel single channel Ca^{2+} currents whose conductance was between those of L- and T-type channels.

Additionally they were insensitive to dihydropyridines which are pharmacological blockers of L-type channels. They were coined N-type calcium channels for their neuronal origin. Not long following this discovery the neuronal non-L-type calcium channels were further classified according to the sensitivity to the cone snail and spider peptide toxins. The channel sensitive to ω-conotoxin GVIA maintained the N-type channel name; however the channel sensitive to ω -Aga IVA toxin was named P/Q-type calcium channel (P for Purkinje cells) and channels resistant to these toxins were termed R-type calcium channels.

2.3.3 Transient Potential Receptor (TRP) channels

The advent of transient receptor potential (trp) research began with the isolation of the Drosophila *trp* mutant by Cosens and Manning in 1969 (Cosens and Manning, 1969). Twenty years passed before the Drosophila *trp* gene was cloned (Montell and Rubin, 1989) and several years later a role for TRP channels was explicitly shown to be essential for the light-activated Ca^{2+} response in Drosophila photoreceptors (Hardie and Minke, 1992; Hardie et al., 1993). Since then there has been an explosion of research literature that has increased exponentially over the past 20 years. At the time of this thesis writing over 21 000 publications and just fewer than 2000 reviews (pubmed search 2015) have been published on TRP channels!

TRP cation channels are described as polymodal cell sensors (Gees et al., 2010; Gees et al., 2012; Nilius and Owsianik, 2011; Wu et al., 2010) mediating physiological responses ranging from sensory physiology (vision, hearing and touch) to homeostatic functions including Ca^{2+} and Mg^{2+} (re)absorption, thermoregulation, as well as pathphysiological responses ranging from night blindness to neurodegenerative as well as kidney disease, skeletal abnormalities and chronic pain (Nilius and Owsianik, 2011; Nilius et al., 2007).

There are 28 different mammalian TRP gene family members (27 in human) that fall into six subfamilies: the “canonical” or “classical” TRPCs (TRPC1-7), the “vanilloid” TRPVs (TRPV1-6), “melastatin” TRPMs (TRPM1-8), the polycystin TRPP (TRPP2, TRPP3, and TRPP5), the mucolipin TRPML (TRPML1-3), and finally the “ankyrin” TRPA (TRPA1). The products encoded by the *trp* genes are intrinsic membrane proteins with six putative transmembrane spans (S1-S6) which contain a cation-permeable pore region between S5 and S6 (Fig. 1). Amongst the most strongly conserved amino acid sequences across the TRP channel family are those flanking the pore region, which encompasses the transmembrane domains S5, and S6 as well as the

TRP domain located on the proximal C terminal tail. The most strongly conserved region spanning 25 amino acids is located in the C-terminal up to S6. Studies using TRP motif mutagenesis with the TRPV1 channel suggests that it may be involved in channel gating (Valente et al., 2008). The intracellular amino and carboxy termini vary significantly between the TRP members as well as the structural domains they encompass (i.e. ankyrin repeats). These cytoplasmic domains are involved in the modulation and regulation of both channel trafficking and function (Owsianik et al., 2006).

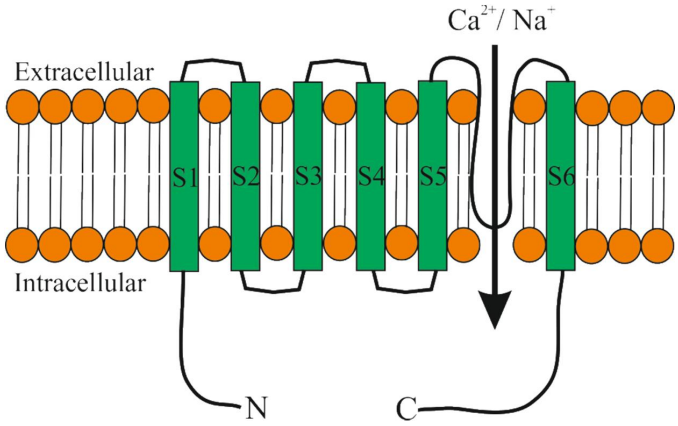

Figure 1 General structure of TRP channels with six transmembrane domains (S1-S6) and cytosolic N- and C terminal tails.

It is now becoming evident that the superfamily of TRPs are emerging as universal mediators of biological sensors, translating a wide variety of environmental cues such as pain perception, mechanosensation, thermosensation as well as taste, phototransduction, and pheromone sensing, into biological signals.

TRP channels can generally be activated either by direct binding of the ligand to the channel, or indirectly via receptor operated production of second messenger that induce channel opening. TRPA1 and the TRPVs, which play a functional role in nociception, can be directly activated by pungent chemicals used by plants to deter them being eaten such as allyisothiocyantes present in mustard (also used as the active ingredient in tear gas), or capsaicin present in chilli peppers. Additionally, molecules that are involved in the sensitization to pain (i.e. hyperalgesia), such as bradykinin present in the inflammatory soup response, are able to modulate channel gating of these nociceptors (Blaustein and Lederer, 1999).

Certain TRP channel family members also play a role in thermosensation, whereby changes in the surrounding temperature can bring about activation by drops in temperatures (below 28 degrees; TRPM8) or signalling in innocuous (warm) and noxious temperature ranges (above 50 degrees) such as the vanilloid TRPs (TRPV1-7) (Clapham, 2003). Some TRP family members are involved in mechanosensation, such as TRPA1. With its long chains of ankyrin repeats with which they provide TRPA1 with spring like protein structures, allows the channel to sense changes in membrane pressure and have been proposed to be ideal candidates for the mechanosensitive protein channels responsible for hearing (Corey et al., 2004).

TRP channels themselves are targets of $[Ca^{2+}]_i$ changes being activated or inhibited by Ca^{2+}. As well as Ca^{2+} dependent activation all TRP channels show an activity dependent inactivation mediated by usually higher concentrations of divalent cations such as Ca^{2+} and Mg^{2+}. Magnesium ions have been shown previously to block a variety of Ca^{2+} permeable channels including members of the TRP channel family (Hardie et al., 1997; Schaffer et al., 2000; Voets et al., 2001). A block by 10mM Mg^{2+} was therefore used as an obligatory criterion in all the statistical analysis done in Article II.

In eukaryotic cells inositol lipid signalling is one of the most extensive signal transduction cascades present. A number of TRP channels, namely the TRPC channels, membrane lipids metabolites can activate or modulate their channel gating properties.

2.3.4 The TRPC subfamily

The TRPC (TRPC1-7) channels were originally discovered as a consequence of research into the mechanisms by which G-protein coupled receptor (GPCR) activation of phospholipase C signalling pathway leads to sustained increases in intracellular calcium (Boulay et al., 1997; Minke and Kirschfeld, 1979; Okada et al., 1998; Zhu and Birnbaumer, 1996). As a result these channels have gathered considerable interest as novel mediators of Ca^{2+} influx for G-protein and second messenger responses.

According to their amino acid sequence homology the TRPCs can be divided into smaller subfamilies: i) TRPC1, ii) TRPC2, iii) TRPC4/5 (approx. 65% sequence homology), and TRPC3/6/7 subfamily (sharing about 70-80% sequence similarity). The TRPC2 protein exists as a pseudogene and is missing from humans. It is expressed in rat and mouse where the role of TRPC2 proteins is in sensing water soluble pheromones (Liman et al., 1999).

TRPCs assemble into tetrameric complexes made up of homomeric or heteromeric subunits. For example via N-terminal coiled-coiled domain interactions homomeric TRPC1 channels are formed. Heteromeric TRPC1-3 interactions can form via the first ankyrin repeat in TRPC1. Additionally TRPC4 and TRPC5 have been shown to interact with TRPC1. TRPC3 has been shown to cooperate with TRPC6 and 7, as well as TRPC1 forming complexes with TRPC3/6 or TRPC4/5 (Clapham et al., 2001)

In the early studies which were searching for stimulators of TRPC channels the diacylglycerol (DAG) molecule which is composed of two fatty acids covalently linked, where effective in activating TRPC3 and TRPC6 (Hofmann et al., 1999). DAGs may be formed from various sources one of which is the GPCR hydrolysis of phosphotidylinositol 4,5-bisphophate (PIP_2). GPCR activation of the Gq phospholipase signalling pathway results in the hydrolysis of PIP_2 resulting in the formation of inositol 1,4,5-trisphosphate (IP_3) and DAG.

Up to the writing of this thesis there are only a few natural occurring compounds that have been described to modulate TRPC channels. Recently it has been shown that the blocking of TRPC channels suppressed the increase in Ca^{2+} entry brought about by 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol (20-GPPD), a metabolite of the ginseng saponin, in CT-26 cells (Hwang et al., 2013). The naturally occurring monoterpene camphor has also been shown to modulate TRPC5 channels after activation of PLC-coupled receptors (Schaefer et al., 2000; Xu et al., 2005). The phloroglucinol derivative hyperforin, the active compound of St. John's wort, was shown to selectively activate TRPC6 (Leuner and Muller, 2006). Nonetheless since TRPC channels are thought to be present as heteromultimeric forms (Li et al., 1999; Peter et al., 2012; Tsiokas et al., 1999) and thus it is and has been very challenging for the community to find compounds that selectively target TRPC channels.

2.3.5 TRPC-NCX

Among the numerous pathways that mediate calcium fluxes, the sodium-calcium exchanger (NCX) has emerged as the predominant mechanism for the efflux of Ca^{2+} across the plasma membrane when intracellular Ca^{2+} concentrations are high (Berridge et al., 2003). With the development of cloning techniques, antibodies and partially purified exchanger preparations the molecular cloning of the canine cardiac Na^{+}/Ca^{2+} exchanger (later on denoted as NCX1) came about (Nicoll et al., 2007). A rapid explosion of sequence information on related molecules followed in the years after with the use of low-stringency library screening. It was established that NCX1 was a

component of a larger gene family SLC (solute carrier) 8, which in itself was part of a larger superfamily of related Ca^{2+}/cation antiporter genes, the CaCA superfamily (Cai and Lytton, 2004). Three members of the SLC8 family are expressed in mammals: NCX1, NCX2, and NCX3.

NCX1 is the mostly broadly expressed member within the SLC8 family. As well as its expression in the heart, it was shown to be highly expressed in the kidney and brain, and lower levels in almost all other tissue (Kofuji et al., 1992; Lee et al., 1994). The other two members of the SLC8 family members have a much more restricted expression pattern. Not significant in most other tissue, the NCX2 is abundantly found in neurons whereas NCX3 is expressed selectively in skeletal muscle and at lower levels in some brain regions (Papa et al., 2003).

The NCX exchanges Na^+ ions for a Ca^{2+} ion at a ratio of 3:1 with two types of transport modes; either the exchange of Ca^{2+} from the extracellular milieu for intracellular Na^+ (hence the influx of Ca^{2+} into the cell) termed the reverse mode, or vice versa, the forward mode whereby intracellular Ca^{2+} is exchanged for extracellular Na^+ leading to an efflux of Ca^{2+} from the cell (Fig.2). The NCX exchanger thus mediates the net transfer of charge as a result of the imbalanced coupling ratio, and is said to be electrogenic since it generates an ionic current. Which mode of transport prevails depends on the electrochemical driving force dictated by the prevailing concentrations of Na^+ and Ca^{2+} as well as the membrane potential (Blaustein and Lederer, 1999).

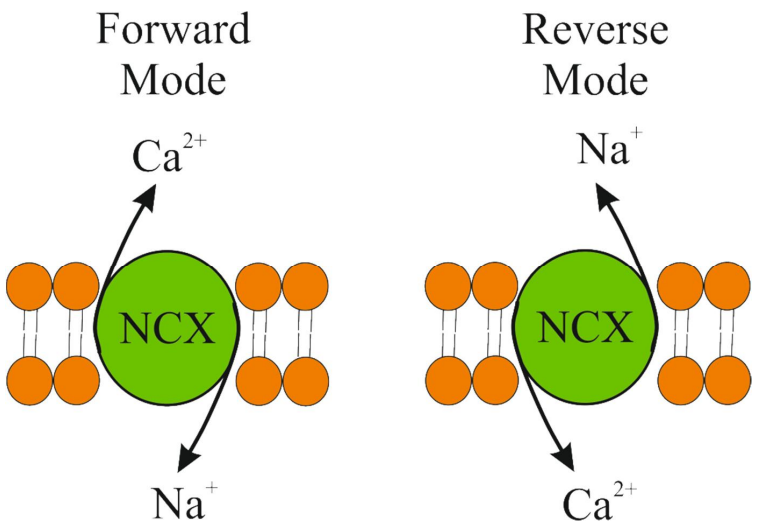

Figure 2 Sodium Calcium Exchanger (NCX) operating in the forward or reverse mode.

It has been suggested that the NCX communicates in close physical proximity with a variety of sodium and calcium transport systems located in both internal stores and the plasma membrane including IP_3 receptors (Solis-Garrido et al., 2004), voltage gated sodium channels (Craner et al., 2004), Na^+/H^+ exchanger (Aiello et al., 2005; Perez et al., 2003), as well as non-selective cation channels (reviewed in (Eder et al., 2005)).

The physical and functional interaction with non-selective cation channels, namely the TRPC3 channel, was suggested in a study using HEK293 expression system (Rosker et al., 2004). The results indicated that TRPC3 enables local accumulation of Na^+ at the cytoplasmic face of the exchanger. This coupled with the resulting membrane depolarization is adequate to drive Ca^{2+} entry via NCX reverse mode (Fig.3).

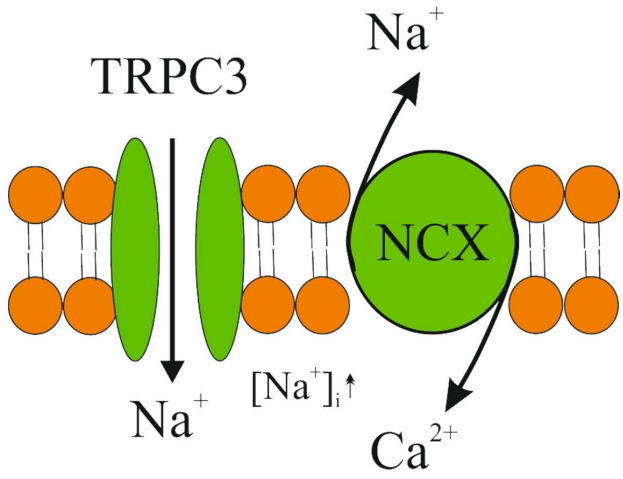

Figure 3 A nonselective cation channel, in this case TRPC3, in close proximity to NCX. The rise in $[Na^+]_i$ causes the NCX to operate in the reverse mode.

2.4 G-protein coupled receptor signalling

The superfamily of GPCR are one of the largest family of proteins in the human genome with more than 800 human GPCR sequences identified (Fredriksson et al., 2003) with an estimation that more than half are targets of most modern drugs (Flowers, 1999).

Common to all GPCRs is a single polypeptide chain with seven transmembrane α-helical domain topology and the ability to interact with guanosine-5'-triphosphate (GTP) binding proteins (G-proteins). All GPCRs are believed to function through a common intracellular mechanism. Agonist binding results in a conformational change in the receptor protein. This in turn leads to the activation of G-proteins by promoting the exchange of the α-subunit's GTP for GDP. Based on the sequence similarities and the profile of activation the α-subunit can be further divided into four subfamilies: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12/13}$. Furthermore, each Gα-family member activates a distinct set of effectors molecules (Oldham and Hamm, 2008).

Once activated the G-proteins modulate the activity of a variety of enzymes, and in turn can also directly or indirectly via second messenger modulate the gating mechanics of ion channels. The commonly accepted effectors via which G-proteins can directly affect include: PLC, adenylyl cyclase, cGMP phosphodiesterase, K^+ channels, and Ca^{2+} channels.

Calcium mobilization by G-protein coupled receptors is most often coupled to the Gq family of proteins. Receptors which are Gq coupled activate a signal cascade where activated Gq activates phosphatidylinositol bisphosphate specific phospholipase C (PLC) to produce inositol trisphospate (IP_3), which binds to the IP_3 receptor in the endoplasmic reticulum (ER) leading to opening of the receptor pore to release stored Ca^{2+} into the cytosol. The released diacylglycerol together with Ca^{2+} activate protein kinase C which causes activation of a variety of signal pathways including those involved in cell proliferation and motility (Fig.4). Protein kinase C can also modify the pattern of Ca^{2+} mobilisation by phosphorylation of TRPC (TRPC3, TRPC6, andTRPC7) channels.

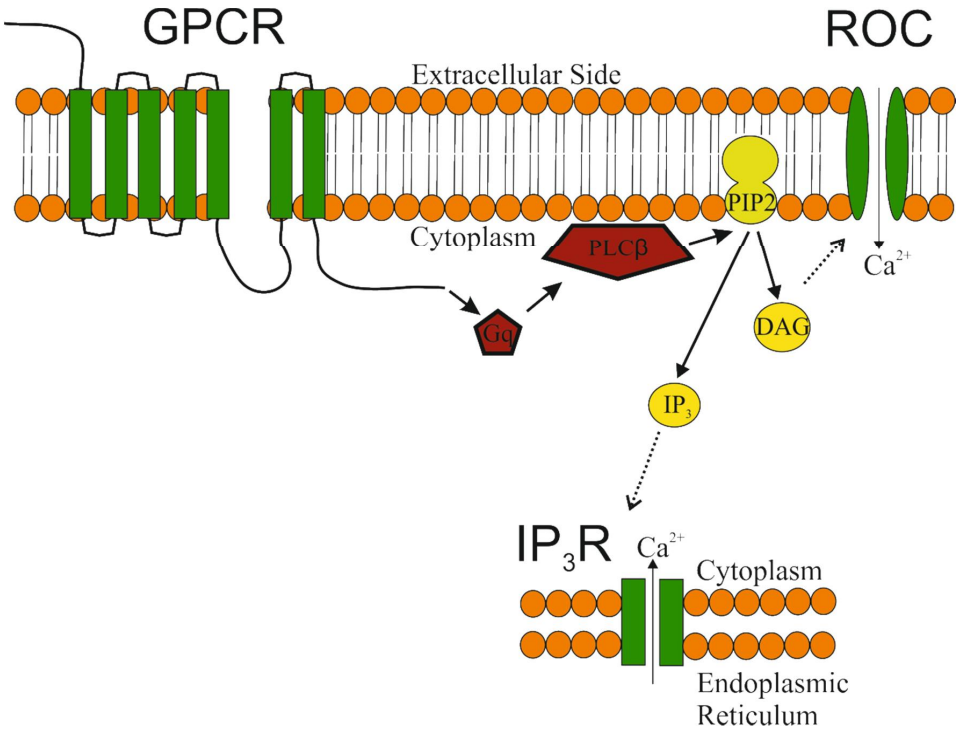

Figure 4 Schematic diagram displaying GPCR signaling via Gq-PLC.

An additional pathway of Ca^{2+} flux is activated during this signalling process. STIM proteins in the ER lumen sense the filling state of the Ca^{2+} store and signals to the plasma membrane to replenish the store via opening of a Ca^{2+} selective channel called Orai (Feske et al., 2006; Liou et al., 2005; Vig et al., 2006; Zhang et al., 2006; Zhang et al., 2005) reviewed in (Hewavitharana et al., 2007) . TRPCs were initially posed as potential candidates for the so called capacitative calcium entry, however many disputed studies were reported where no clear role was shown demonstrating TRPC channels as mediators of the store operated calcium fluxes and currents. Also other G-proteins are able to activate Ca^{2+} mobilisation. Namely subunits from G_i family proteins are also able to activate PLC (Yada et al., 1989; Yang et al., 1991). Furthermore cAMP, produced via G_s coupling and adenylyl cyclase activation activates in some cells the so called cyclic nuclide gated and hyperpolarization-activated cylicnulceotide channels which are calcium and sodium permeable channels in the plasma membrane.

2.5 Orexin GPCR signaling couples to non-selective calcium permeable channels

The orexin receptors were found by screening the effect of hypothalamic extracts on orphan G-protein coupled receptors called HFGAN97 using calcium mobilization as a read out method (Sakurai et al., 1998). The purified active peptides were called orexin A and orexin B and the receptors Ox1R and Ox2R. Since the receptors were coupled to Ca^{2+} elevations in cells it was assumed that they would depend on $G_q \rightarrow PLC \rightarrow IP_3 \rightarrow Ca^{2+}$ release (Sakurai et al., 1998). Instead it was found that the receptors when stimulated with low concentrations of orexins activated a pathway for calcium influx which was independent on Ca^{2+} release from stores (Lund et al., 2000).This signaling pathway is different from that seen in classical PLC-Ca^{2+} release pathway which when activated would induce store operated Ca^{2+} channels and/or calcium release activated calcium channels (Orai) with respect to effects of blockers and modulation by protein kinase C (Larsson et al., 2005). The Ca^{2+} influx through this pathway was inhibited by dominant negative constructs of TRPC3 channels (Larsson et al., 2005; Nasman et al., 2006). At higher concentrations of orexins coupling to the typical $G_q \rightarrow PLC \rightarrow IP_3 \rightarrow Ca^{2+}$ system is seen (Lund et al., 2000; Magga et al., 2006). Orexin induced Ca^{2+} release from stores has so far not been reported in native orexin receptor expressing neurons. Instead activation of nonselective cation channels have been reported in several studies (Eriksson et al., 2001; Keele et al., 1997; Lee and Boden, 1997; Smith and Armstrong, 1996). Orexin activated a voltage dependent calcium influx pathway whose primary depolarization was a result of an unidentified nonselective cation channel has also been observed (Kohlmeier et al., 2008). Remarkably the original work by Eriksson demonstrated that the sodium-calcium exchanger (NCX) blocker KB-R7943 could partially block currents activated by the orexin receptor suggesting a role of NCX in responses to orexin in neuros. A residual current was left that was sensitive to Nickel which is known also to block non-selective calcium permeable channels. Secondly, it was shown some years later on that the blocker widely used to block NCX action at higher concentrations (such concentrations used in slice preparation studies) also inhibited the non-selective TRPC channels. The mechanism by which orexin activates neurons is thus not fully clarified.

2.6 TRPC channels in neuronal development

Accumulating evidence illustrates that changes in intracellular calcium concentrations have an important and diverse role in the response to extracellular guidance cues, such as brain derived neurotrophic factor (BDNF), as well as in regulating the motor-motility machinery driving migratory processes (reviewed in (Zheng and Poo, 2007)). The target of BDNF action acts on a specific subpopulation of migrating neurons (Jansson et al., 2012; Ohmiya et al., 2002; Polleux et al., 2002). We recently showed that the increase in motility brought about by BDNF effects a subpopulation of cells of a Calbinding and NeuN positive cell population, reducing the phases of slow movement and prolonging high motility phases (Jansson et al., 2012). Chemoattractive action of BDNF is thought to increase cytosolic concentrations of calcium by triggering Ca^{2+} influx pathways and Ca^{2+} discharge from intracellular stores. Despite detailed studies on BDNF and its role in cortical development information is lacking on the molecular mechanisms responsible. *Trpc3* mRNA and TRPC3 channel proteins compared to other TRPC channels have a particularly high expression in embryonic tissue (Strubing et al., 2003). The tyrosine kinase receptor; BDNF, has been shown to activate a nonselective cation channel whose current was shown to be contributed by TRPC3 channels (Amaral and Pozzo-Miller, 2007; Li et al., 1999) in hippocampal cells. RNAi targeted against *Trpc3* interferes with BDNF-induced turning of axons (Aiello et al., 2005). In addition to typical chemotactic substances TRPC3 channels in the nervous system are also activated by Group I metabotropic glutamate receptors. Nevertheless, studies on the role of TRPC3 in corticogensis are limited as well.

3. Aims of the Study

The aim of the thesis was to elucidate the role of calcium influx pathways and their coupling to membrane events, namely via endogenously expressed TRP and VGCC channels, in the differentiation and migration of neuronal cells. The more specific aims of this work are as follows:

1) In order to study the effect of cell differentiation on endogenous TRP channel expression, the IMR-32 neuronal cell model system was used and functional studies were carried out to assess TRP channel function (I)

2) It has been previously shown that certain G-protein coupled receptors (orexin & metabotropic glutamate receptors) couple to novel calcium influx pathways involving TRPC channels; however the signaling pathways leading to the rise in intracellular Ca^{2+} was not fully elucidated. To study the role TRPC channels in receptor operated calcium signaling, TRPC channel gene expression in IMR-32 cells was disrupted and functional studies via stably transfected orexin receptors as well as endogenous bradykinin receptors were carried out (II).

3) As well their mediation of novel calcium influx pathways coupled to G-protein coupled receptors, TRPC channels have been suggested to play a role in mediating migratory guidance ques. Nonetheless studies on their role in corticogenesis are limited. Using an *in vitro* neurosphere assay model and comparing WT vs TRPC knockout mice derived neural stem cells we set out to clarify their physiological significance in neuronal migration and differentiation (III).

4) Another calcium influx pathway shown to be involved in neuronal migration are the voltage gated calcium channels. Most of the studies previously were done on postnatal neurons, and thus their role in early cortical development is less well investigated. Using an *in vitro* neurosphere assay model we studied the expression and functional significance of VGCC in neural stem cell differentiation and migration (IV).

4. Materials and Methods

4.1 IMR-32 cell culturing and differentiation (I-II)

The neuroblastoma cell line IMR-32 (Tumilowicz et al., 1970) was obtained from the American Type Culture Collection. This adrenergic human cell line respond to treatment with 5-Bromo-2'-deoxyuridine (BrdU) by acquiring a neuronal phenotype, extending long axon –like processes with numerous growth cones as well as exhibiting a wide number of voltage gated calcium channels (Carbone et al., 1990; Clementi et al., 1986). The cells were grown in standard Minimum Essential cell culture medium with 10% fetal bovine serum and 100units/ml penicillin-streptomycin. The cells were grown in 80cm^2 cell culture flasks at 37°C in a humidified air ventilated incubator (95% air/5% CO_2). For experiments, cells were seeded onto square coverslips for electrophysiological measurements, or round coverslips for calcium imaging, in tissue culture dishes. Differentiation was induced by introducing BrdU at a concentration of 5μM the day after seeding. The cell culture medium was exchanged with fresh medium containing BrdU every 2 to 3 days. Experiments were performed 8-12 days later.

4.2 Mice (III-IV)

The mice were housed in qualified animal facilities with standard laboratory conditions which are in accordance with National Institutes of Health guidelines. Two different inbred strains of mice Friends virus B (FVB) (III, IV) and C57BL/6 (III) were used. Both strains are commonly used in construction of genetically engineered mice to assess specific gene function. We have previously characterized quite extensively the neural progenitor cell *in vitro* from FVB mice (Castren et al., 2005; Jansson et al., 2012; Louhivuori et al., 2011) and thus was a natural step to continue their characterization in paper III. The thesis project focused on the function of canonical transient receptor channels (TRPC) in neural progenitor migration and due to the current lack of TRPC specific channel blockers led us to collaborate with Prof. Veit Flockerzi (Homberg Germany) and gain access to TRPC gene deficient C57BL/6 mice (III). The gene deficient mice used were TRPC3,-6 double knockout (TRPC3/6 DKO) and TRPC1, -4, -5 triple knockout (TRPC1/4/5 TKO). The gene deficiency is located in exon 7 for TRPC1, TRPC4, TRPC5, as well as TRPC3 and TRPC6, resulting in the excision of the pore region of the channel.

4.3 Neural Progenitor Cell Preparation and culturing (III and IV)

Pregnant female mice were anesthetized with CO_2 and sacrificed by cervical dislocation followed by the hasty removal and sacrifice of the pups at embryonic day 14 (E14). Neuronal stem cells (NSC) were generated from the wall of the lateral ventricles as described in detail by Clarke et. al. (Clarke et al., 2000). Briefly, the tissue was dissociated in Hank's balanced salts solution (HBSS) containing 2 mM glucose, 0.7 mg/ml hylauronidase, 0.2 mg/ml kynurenic acid and 1.33 mg/ml trypsin for 30 min at 37°C. After titration the cells were centrifuged at 1500 rotation per minute (rpm) (equivalent to ~500g) for 5 min, resuspended in 0.9M sucrose in HBSS, and centrifuged for 10 min at 2000 rpm (~900g). The cells were then resuspended in 2 ml of Earle's balanced salt solution (EBSS), titrated carefully on 10ml EBSS containing 4% bovine serum (BSA) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) then centrifuged for 7 min at 1500 rpm. The cell pellet was resuspended and plated in culture flasks containing Dulbecco's Modified Eagle Medium: Ham's Nutrient Mixture F-12 media (1:1) with B27 supplement, 2mM L-glutamine, 15 mM HEPES, 100U/ml penicillin and streptomycin, 20ng/ml EGF and 10ng/ml EGF, and placed in a humidified incubator (5% CO_2, 95% air). Cells were grown as free-floating aggregates, known as neurospheres. The culture media was refreshed twice a week and growth factors were added three times per week. Cells were passaged by manual trituration at approximately 7-10 day intervals.

4.4 Neural Progenitor Cell Differentiation (III and IV)

Employing the neurosphere assay (Reynolds et al., 1992), the NPC aggregates (200-250 μm) were plated on poly-DL-ornithine (Sigma) coated culture dishes or cover glasses in the absence of EGF and FGF. Removal of mitogen leads to the spontaneous differentiation and migration of neuroblasts and radial glial processes from the neurosphere. To study the effects of voltage gated calcium channel blockers on neural differentiation (III) the low voltage channel blockers NNC 55-0396, mibefradil, pimozide, ethoxsumide, $NiCl_2$ or the high voltage channel blockers nifedipine, ω-conotoxin were added to the differentiation media before or 1-3 days after plating. In study IV the effects of the trpc3 channel blocker pyr3, and the chemoattractant BDNF were added to the differentiating media before plating of the neurospheres.

4.5 Immunocytochemistry (I, III and IV)

The cells on covers slips were fixed using 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS, pH 7.4) for 20 min at room temperature. The cells were then permeabilized and blocked for non-specific staining for 60 min at room temperature. Cells were incubated with primary antibodies (see articles for detailed lists of antibodies used) and incubated overnight at 4ºC. Secondary antibodies were applied for 1h at room temperature in the dark in 1% BSA-PBS. The nuclei of the cells were counterstained with 4'6-diamino-2-pheylindole (DAPI). For secondary antibody controls primary antibodies were omitted, resulting in the disappearance of all staining. Cells were viewed and photographed using either LSM 5 Pascal system (Carl Zeiss) or an Olympus AX70 Provis microscope (Olympus) with x10 (numerical aperture 0.30) or x20 (numerical aperture 0.50) object magnification, equipped with fluorescence optics and a charge-coupled device camera (PCO AG).

4.6 Gene Expression Analysis (I, III and IV)

4.6.1 RNA isolation and cDNA synthesis

IMR-32 cells

Using Trizol (Invitrogen) RNA was extracted from non-differentiated and 8 days differentiated cells, then according to the manufacturer's instructions using 5mg total RNA were reverse transcribed with Superscript first strand synthesis kit (Invitrogen). RT-PCR products were run on 1.5% agarose TBE. After completion of the run the gel was stained with ethidium bromide and image captured with Bio-Rad Gel Doc 2000. In order to identify the PCR products, they were gel purified and inserted into PGemTeasy T-vectors. Sequencing was done using MegaBACE 750 (GE Healthcare, Uppsala, Sweden) DNA Analysis System.

Neural Progenitor Cells (III, IV)

The total RNA from non-differentiated NPCs and differentiated (for 1 and 5 days) NPCs were isolated according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen). Quantification was done using NanoDrop ND8000 spectrophotometer

(Thermo Scientific). Using the Transcriptor High Fidelity cDNA Synthesis Kit and random hexamer primers complementary deoxyribo-nucleic acid (cDNA) was synthesized according to the manufacturer's instructions (Roche Applied Science).

4.7 Calcium Imaging (I-IV)

The Ca^{2+} imaging experiments (I-IV) were performed and the data were analyzed by using the intracellular imaging InCyt2TM fluorescence imaging system (Intracellular Imaging). The cells were perfused with Hepes buffered media (HBM) at 37 °C and excited by alternating wavelengths of 340 and 380 nm by using narrow band excitation filters. The emitted fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera. One ratio image was acquired per second. Fluorescence from 340 and 380 nm exposures were imported into Microcal OriginTM 6.0, and the ratios (340/380) were calculated.

4.8 Electrophysiology (I, III and IV)

Whole cell patch clamp recordings were measured in voltage clamp mode or current clamp mode at 28°C by using the standard whole-cell configuration (Hamill et al., 1981). The cover slip with the attached cells were attached to the bottom of an RC-24 fast exchange chamber (Warner Instruments Inc.) and positioned on top of the microscope. Cells were perfused with HBM by a gravity-controlled drug delivery system. The perfusates were converging in a perfusion manifold and funneled through an SH-27B in-line heater (Warner Instruments Inc.) located just before the chamber inlet to obtain the desired temperature. Patch pipettes (model PG150T, Harvard Apparatus) were prepared with a PC-10 puller and flame-polished in a microforge MF-900 (Narishige) to a resistance of 3.6 – 3.8 megaohms measured in the bath solution. The patch clamp amplifier Axopatch 200A was connected to a computer via the AD/DA Digidata 1320E SCSI interface (Axon Instruments). Voltage protocols and data acquisition were controlled with pClamp 8.1 (Axon Instruments). Cells were compensated for the pipette capacitance, whereas following whole-cell access, the series resistance was analogically compensated to 60 – 70 %. Liquid junction potential was calculated using pClamp8.1 and subtracted from the recordings giving a more accurate clamping potential. Data was digitally sampled at 3.8 kHz and filtered at 2 kHz by using the low pass Bessel filter on the recording amplifier.

4.9 Time-lapse Imaging (Cell-IQ®) (III and IV)

The Cell-IQ system (Chip-Man Technologies Ltd.) is a self-contained cell culture instrument which combines phase contrast microscopy, automation, and environmental control enabling the continuous monitoring of adherent cells which are plated on plates mounted on an integrated plate holder. The instrument contains an integrated incubator (±0.2ºC), precision movement stages (x, y axes: ±1 μm; z axis: ±0.4 μm), two incubation gas flow controllers, and an automated optics module. The collected images are were then processed and analyzed with Image-J software.

5. Results and Discussion

Two *in vitro* cell model systems were employed in the thesis; the human IMR-32 neuroblastoma cell line, which when treated with BrdU, undergoes a striking functional and morphological differentiation into mature neuron-like cells (Carbone et al., 1990) (I, II). The second is the neural progenitor stem cell neurosphere assay where by mitogen removal from the culturing media induces the differentiation and migration of radial glial cells and neuronal cells (III, IV).

The IMR-32 cells were chosen due to a number of reasons. Firstly, upon differentiation, the human neuroblastoma cell line undergoes dramatic changes in both morphology and gene expression. Upon treatment with BrdU the IMR-32 cells acquire a neuronal phenotype displaying a clear morphological change seen with the outgrowth of long axon-like extensions as well as acquiring N- and T-type VGCCs (Carbone et al., 1990; Clementi et al., 1986). Furthermore differentiated IMR-32 cells express mRNA for *Trpc*1, *3-7* (Nasman et al., 2006). In addition to *Trpc* channel mRNAs, we found that the IMR-32 cells express mRNAs for several members of the *Trpv* (*Trpv1*, *2*, *3*, *4*, and *6*), and *Trpm* families (*Trpm1, 3, 6, 7*) (Paper I: Supplementary Fig. S4). In addition in article I we show that these cells express mRNA for the cold sensitive channels TRPA1 and TRPM8. Secondly, they were chosen for their transduction efficiency with modified baculovirus-based vectors allowing the manipulation of TRPC channel proteins via targeted mRNA disruption. Thirdly, our extensive studies on orexin signaling in IMR-32 cells stably expressing the Ox1R as well as their endogenous expression of the bradykinin receptors whom like orexin receptors are also coupled to PLC, provided an ideal cell model to investigate metabotropic GPCR signaling and its coupling to TRPC channels and calcium elevations.

5.1 TRP channels upregulated in differentiation of neuronal cells: TRPA1 and TRPM8 (I)

The TRP channels being nonselective cation channels permeable to cations result in cell depolarization when activated. TRP channels being polymodal (as described in the Introduction) can be activated directly (i.e. via pungent chemicals) or coupled to receptor operated channels. In our initial characterization of TRP channel expression in IMR-32 cells we surprisingly found that TRPA1 showed a dramatic increase in detectable mRNA when IMR-32 cells were differentiated for 8 days compared to non-differentiated cells (paper I: Fig.1). Supporting the mRNA data, the immunoreactivity of TRPA1 was not detected in non-differentiated cells however 20–30% of differentiated cells were positive. This finding as well as the abundance of ligands known as activators of this channels gave us a unique opportunity to investigate TRPA1 channels in an endogenous environment using an established neuronal cell model.

Using whole cell current clamp recordings performed with potassium based pipette solutions we examined the effect of TRPA1 activation on the cell membrane. The TRPA1 agonist wasabi (a Japanese horseradish) caused a depolarization of 19.6 ±7.3mV at a resting membrane potential of 50.9 ±5.0mV (n=5/10). Non-differentiated cells where *Trpa1* mRNA was not detected did not show any response to wasabi (n=10). This was similarly seen when TRPM8 channels were directly stimulated with menthol and displayed strong outward rectification (resulting in cell membrane depolarization).

After we had confirmed that differentiated IMR-32 cells express functional TRPA1 channels by using known pungent agonists against the channel like wasabi and AITC (allyl isothiocyanate), and established that the electrophysiological characteristics are similar to those published previously (Nagata et al., 2005) we directed our attention on cold activation. The first cold-sensing TRP channel candidate was TRPA1. In the PNS, this channel is expressed in a subset of capsaicin sensitive positive neurons with an activation threshold of 17ºC (Story et al., 2003).

Our results in paper I does not support a thermosensory function of TRPA1 in neuroblastoma cells. Our findings failed to obtain a correlation in the calcium responses between cooling (<15ºC) and TRPA1 activation (AITC). Since we found mRNA for *Trpm8* we investigated whether it could account for the calcium response elicited by the noxious cold stimulation. Recent studies that have removed TRPM8-positive neurons through genetic interference have highlighted their role in noxious cold perception in mice (Pogorzala et al., 2013). Noxious cold is considered to occur at temperatures below 15ºC. TRPM8 is activated by temperatures below 25ºC (i.e. innocuous cool and noxious cold range) and activated by the cooling compound menthol (McKemy et al.,

2002). TPRA1 and TRPM8 expressing neurons show a lineage relationship with TRPV1 expressing neurons. TRPV1 is generally considered a marker for nociceptors and during development is expressed in TRPM8 positive neurons. Conditional knockout of TRPV1 sensory neurons caused deficits in responses to acute noxious cold (Mishra et al., 2011).

Even if they expressed mRNA for Trpv1 differentiated IMR-32 cells did not respond to capsaicin, an activator of TRPV1 channels (Paper I). Of all the cells recorded, around 12 % of the cells gave a calcium response to 200 uM menthol, from which 90% of the cells responded to AITC. Within the cold sensitive population ~10% responded to menthol whilst around 40% responded to AITC. Twentyseven percent of the cells that displayed a calcium response to cooling no observable response to AITC or menthol was recorded. This is to some degree similar to the study done by Jordt (Jordt et al., 2004) which found that the majority of cultured trigeminal neurons from rat (96%) which were sensitive to the TRPA1 agonist AITC were insensitive to cold, whereas the remaining 4% were sensitive to menthol.

Despite the vast amount of research directed at TRPA1, whether and how it is activated by temperature was quite unclear during the work on paper I (Bang and Hwang, 2009; Caspani and Heppenstall, 2009; Kwan and Corey, 2009). Specifically the question whether peripheral TRPA1 channels can serve as cold sensors did not produce any definitive answer. Since it was first shown in early 2003 by Story et. al. (Story et al., 2003) a number of studies have supported the finding that TRPA1 plays a role in the sensation of noxious cold (Andersson et al., 2008; Bandell et al., 2004; del Camino et al., 2010; Fajardo et al., 2008; Karashima et al., 2009; Klionsky et al., 2007; Leung et al., 2006; Viswanath et al., 2003). Nevertheless, a number of studies have contradicted this notion (Bautista et al., 2006; del Camino et al., 2010; Jordt et al., 2004; Knowlton et al., 2011; Kwan and Corey, 2009; Nagata et al., 2005).

Interestingly cold sensory mechanisms in neurons are observed during embryonic development long before expression of TRPA1 or TRPM8 is measurable (Hjerling-Leffler et al., 2007). It is therefore likely that in differentiated IMR-32 cells (and probably sympathetic neurons) the TRPA1 channel does not function primarily as a thermosensor. Our findings have recently been supported by knockout studies in rodents (de Oliveira et al., 2014). A most likely candidate for the cold thermosensation in the subpopulations of cells that lack correlation with TRPA1 and TRPM8 may reside in the two pore tandem potassium channel family TREKs and TRAAKs, however this warrants further investigation. Several studies done, during the writing of this thesis, point towards this hypothesis (Morenilla-Palao et al., 2014; Pereira et al., 2014).

The physiological role of TRPA1/TRPM8 channels in differentiated IMR-32 cells is not obvious. The catecholaminergic characteristics of IMR-32 cells are apparently derived from neuroblasts developing into sympathetic neurons (Gotti et al., 1987; Tumilowicz et al., 1970). Interestingly we have previously shown that TRPA1 activation of the neuroendocrine STC-1 cells regulates cholecystokinin release (Purhonen et al., 2008) suggesting that pungent ingredients in our diet can play a role in intestinal function. Furthermore in the CNS a novel role for TRPA1 channels has been shown to be in important in the regulation of the GABA transporter GAT-3 by modulating resting intracellular calcium levels of astrocytes by giving rise to highly localized near membrane calcium microdomains (Shigetomi et al., 2012).

TRP channels themselves are targets of $[Ca^{2+}]_i$ changes being activated or inhibited by calcium (Article I). Our work with TRPA1 clearly demonstrated that intracellular calcium potentiated the response. In the absence of extracellular Ca^{2+} whole cell channel flickering was recorded illustrating channel activation (Article I: Fig. 5C). Readdition of Ca^{2+} to the extracellular recording solution potentiated the current (Article I: Fig. 5C, D). Additionally, wasabi slightly depolarized the membrane of differentiated IMR-32 cells in the absence of extracellular Ca^{2+} however when Ca^{2+} was added back to the bathing media the depolarization was potentiated (Article I: Fig. 5E).

5.2 G-protein coupled receptors activate TRPC: local modulation of intracellular sodium which locally induce NCX into reverse mode (II)

Despite the extensive studies into TRPC signaling there have been a few reported cases on the specific roles of endogenously expressed TRPC channel function in the nervous system. The mechanisms by which TRPC channels are activated by G-protein coupled receptors and by which they regulate intracellular free calcium are far from clear. TRPC activation in neurons is usually associated with Ca^{2+} entry into the cells and the elevation of intracellular Ca^{2+} (Talavera et al., 2008). However in neurons it remains uncertain whether Ca^{2+} entry is the primary response to TRPC activation. TRP channels as well as being a primary source of some Ca^{2+} influx (calcium ions flowing through the open pore) cause a substantial Na^{+} influx and subsequent membrane depolarization This can modulate other channels that are sensitive to small changes in membrane depolarization (such as low voltage activated calcium channels: See below; Article IV) as well as by changing the calcium/sodium concentrations locally, affect Na^{+} gradient and membrane potential regulated transporters such as NCX in close association with the TRP channel, leading to changes in intracellular Ca^{2+}. Blockers of voltage gated calcium channels do not affect the calcium entry in response to orexin A acting on Ox1 receptors (Nasman et al., 2006). Therefore we investigated to what extent reversal of NCX pathways contribute to Ca^{2+} elevations seen upon stimulation of transduced Ox1R and endogenous bradykinin receptors.

In Article II we found that stimulating neuroblastoma cells with the GPCR ligand bradykinin resulted in a Na^{+} dependent depolarization. A depolarization linked to Na^{+} influx and a local increase in intracellular Na^{+} would lead to a reversal of sodium/calcium exchange towards influx of calcium (Blaustein and Lederer, 1999). Using dominant negative and ShRNA we showed that the Ca^{2+} responses to Ox1R and bradykinin receptor stimulation were partially dependent on the function of TRPC3 channels. The most prominent electrophysiological occurrences recorded in TRPC3 expressing cells is a depolarizing Na^{+} inward current of whose functional significance has mostly been neglected. Thus stimulation of receptors coupled to PLC in cells over-expressing TRPC3, large inward Na^{+} currents are measured during stimulation. A classical procedure to show the involvement of the sodium calcium exchanger in mediating calcium responses is to disturb its function by substituting sodium ions with the impermeable ion NMDG. A natural candidate would be the calcium permeable TRP channels.

When sodium was removed Ca^{2+} entry was blocked in a population of the cells. When Na^{+} was replenished the cells could be classified as either Na^{+}-dependent (those whose

calcium response was significantly reduced) or Na^+-independent (whose calcium response were either unaffected or slightly enhanced) (Article II; Fig.I). Furthermore the Na^+-dependent Ca^{2+} influxes were efficiently blocked by PKC activation, by NCX inhibitors, and by molecular disruption of TRPC3/6 channel function. On the other hand, the Na^+-independent Ca^{2+} responsive cells were not affected by the above mentioned treatments, but were sensitive to polyvalent cations, such as spermine, Gd^{3+} and ruthenium red (Article II; Fig.3b, Fig. 5C and D, and Fig.6C). We also found that the Na^+-dependent response largely disappeared upon knockdown of TRPC3/6 channels by co-expression of the dominant negative TRPC6 constructs and shRNA against TRPC3. Thus the data suggests that a NCX-TRPC channel interaction constitutes an important functional unit in receptor-mediated Ca^{2+} influx.

In agreement with our data reducing the extracellular calcium concentration in previous studies on neurons stimulated with orexin led to in most cases to an inhibited calcium response to Ox1R stimulation (Ishibashi et al., 2005; Kohlmeier et al., 2004; Nakamura et al., 2010; Uramura et al., 2001; van den Pol et al., 1998). Nonselective cation channels were identified as one of the major mediators of orexin induced depolarization in CNS neurons (Brown et al., 2002; Burlet et al., 2002; Liu et al., 2002; van den Pol et al., 2002; Yang and Ferguson, 2002) however their identity remained elusive. Complicating the matter were other mediators in number of different native neurons were implicated including the sodium/calcium exchanger, potassium channels and voltage gated calcium channels (reviewed in (Kukkonen and Leonard, 2014)).

TRPC channels do not respond to a single stimulator per se, but rather to multiple different factors resulting in the opening of the channels. TRPC channels are voltage sensitive (membrane voltage can change ion conductance properties) and not voltage dependent (i.e. requiring a change in membrane potential to open). They may show constitutive activity and may be stimulated or inhibited by a range of chemical and protein factors. The diversity in channel activation suggests that these channels may exist as integrative sensors of complex chemical signals and represent an important class of channel proteins mediating receptor operated calcium influx.

5.3 TRPC channels: their role in the early differentiation stages of migrating neural progenitor cells (III)

The neurosphere culturing method provides an assay whereby cell distribution can be quantitatively analyzed using imaging methods. The mother sphere functions as a point of origin and the emanating cell populations can be mapped spatially with regards to this point. This characterization method has been imperative in all subsequent studies using the neurosphere assay since it provided us with tools to dissect the heterologous populations. Till today in the time of writing this thesis, specific antibodies to identify the subpopulations of cortical progenitor cells in the developing brain are lacking, albeit progress is in the right direction. These findings of spatial correlation with the immunostaining results and functional calcium responses echo in both studies used in this thesis (Article III and IV). From the initial stages of differentiation the radial glial marker GLAST (Hartfuss et al., 2001) was expressed by cells emerging from the neurospheres (III and IV), as seen in Article IV Figure 1. These GLAST positive cells with the passage of time form a dense layer of cells around the neurosphere. Closer inspection of the distribution of GLAST expression, a strong fluorescent intensity was measured at the tips of the individual radial glial processes (III: Figure 5A).

The microtubule associated protein (MAP) -2 (III, and IV), which is a marker for neuronal cells (Dehmelt and Halpain, 2005) became most evident after 24 hrs of differentiation. The morphology of the cells typically had small cell bodies and thin protruding processes. The neuronal marker expression was highest in cells outside the radial glial cell layer (IV: Fig.1) with MAP-2 immunostaining also present on and between the radial glial processes.

Due to the potential role of TRP channels as chemosensors and reported guidance mediators of extracellular cues (Tai et al., 2009), we proceeded to analyze the role of TRPC channels on NPC migration. Our mRNA data demonstrated that there was a high expression *Trpc1* and *Trpc3* mRNA in differentiating NPCs compared to *Trpc4-7* which were not clearly detected. This is in line to some degree with previous studies on *Trpc* mRNA expression at E13 in the embryonic cortex which showed the expression of *Trpc1/3* and *5* with low to barely detectable expression of *Trpc4/6/7* (Boisseau et al., 2009).

Upon initiating this study the main problem in studies on the role of TRPC channels was the lack of specific pharmacological tools to functionally identify channels in question. We therefore set out in paper III to elucidate the function of TRPC channels in early stages of NPC development using TRPC knockout mice. Cells were prepared from mice lacking functional TRPC3/6 and TRPC1/4/5. During the course of the study

a selective blocker for TRPC3 channels pyr3 was reported. This allowed us to compare data from knockouts and inhibitor treated cells.

5.4 Disrupting TRPC channel function increases neural progenitor cell motility by reducing stalling duration and turning frequency (III)

Time lapse microscopy was used to visualize the migratory behavior of NPCs. It was observed that neuron-like cells exiting from radial glial layer routinely changed directions while migrating, alternating between fast bursts of rapid movement and intermittent stationary pauses. These observations are consistent with previously described subventricular zone neurons migrating in slices (Murase and Horwitz, 2002; Suzuki and Goldman, 2003; Ward et al., 2005). Due to the phasic motility pattern the average speed of movement may not be an appropriate parameter for analyzing cellular motility. In order to statistically analyze the data we developed an index which we coined the motility index. This was defined as the number of time points the cell moved with a speed higher than 40 μm/h divided by the number of time points the speed of the cell was below this value at a time period of at least 10 hours.

A closer look at the behavior of the cells during their motility provided further parameters to investigate the effect of TRPC channels on migrating NPCs. During the intermittent phase (i.e. stalling) the cells are not totally immobile but rotate with a low speed and extend a number processes (Article IV: Fig.1B) and a change the direction of movement with the subsequent burst of movement. Studies on developing *xenopus* neurons suggest that during stalling periods, the cells a) explore their surrounding environment and b) cause robust calcium elevations (Gomez and Spitzer, 1999).

Using TRPC KO models (TRPC3/6 KO and TRPC1/4/5 KO) an interesting pattern appeared. Firstly since we hardly have detectable levels of mRNA for *Trpc4-7* the knockouts in this stage of development are affecting TRPC3 or TRPC1 channels. Disrupting either TRPC3 or TRPC1 channel function caused a significant increase in the motility index compared to their control WT (Article III; Fig.2E). This would suggest that both channels are involved in promoting the stalling phase. However, cells from the two knockouts showed a marked difference in their turning frequency. TRPC1/4/5 KO cells displayed no statistical difference compared to WT values, whilst TRPC3/6 KO (and blocking of the active TRPC3 channel with pyr3) had considerably less turning points. Since treatment of cells with, MPEP, a blocker of metabotropic glutamate receptor 5 (mGluR5) had a similar effect on the turning frequency (Jansson et al., 2013) and since the expression of mGluR5 dominates in radial glia (Jansson et al.,

2012) we analyzed the interaction of the neuronal cells with radial processes. We found that wild type cells made frequent contacts with radial processes and this interaction was disrupted in the TRPC3/6 KO cells. The TRPC3/6 KO NPCs had nearly twice the amount of free cells compared to wild-type (Article III: Fig.6).

5.5 Role of radial glial processes and mGluR5 in neuronal motility (III)

TRPC3 channels are activated by mGluR5 (and mGluR1) (Berg et al., 2007; Kim et al., 2003). We stimulated NPC to DHPG and obtained calcium responses sensitive to the TRPC3 blocker pyr3 (Article III: Fig.4A). TRPC3/6 KO cells lost this sensitivity to pyr3 inhibition (Article III: Fig.4B). Curiously there was no difference in the average amplitude of the DHPG calcium response between the KO and WT cells, suggesting that compensatory mechanisms are involved e.g. some other channels compensates for the lack of TRPC3 with respect to the global Ca^{2+} response in the TRPC3/6 KO cells but do not compensate for the effect on motility.

There is a substantial amount of data supporting the fact radial glial can influence neuronal cell motility, as well as axonal growth and synapse formation by either factors produced by radial glial cells and/or on direct contacts made between them (reviewed in (Sild and Ruthazer, 2011)). Investigating the radial glial morphology in TRPC3/6 KO cells, a clear distortion was observed (Article III: Fig.) similar to the effect MPEP had on the radial glial cell morphology (Jansson et al., 2012). Furthermore the stalling frequency of the cells coincided with the distance to the radial glial cells, with cells displaying longer stalling times when near the radial glial network. This was disturbed in the TRPC3/6KO cells, as well as in WT cells under the block of MPEP or pyr3 (Article III: Fig.7).

Therefore taking into account the mGluR5 distribution and the similarity of the motility response to MPEP and to interference with TRPC3, the block of Ca^{2+} influx in response to DHPG by pyr3 and that both interference with TRPC3 function and MPEP distort the structure of radial processes would suggest that mGluR5 acting via TRPC3 is plays a central role in the communication pathway between radial glia and neuronal cells.

5.6 TRPC channels and their function in BDNF induced motility (III)

BDNF has previously been shown to activate TRPC3 channels in several studies (Amaral and Pozzo-Miller, 2007; Li et al., 1999). As in earlier studies (Jansson et al., 2012) and in Article III: Fig.3 BDNF increased the motility index of the neuronal progenitor cells compared to WT, however it did not increase the motility index of the TRPC3/6 KO mice. This would suggest that TRPC3 channels are involved in the BDNF promoted motility increase. On the other hand, TRPC1/4/5 KO mice BDNF significantly enhanced further the already augmented motility index.

TRPC1 channels have been reported to be mainly expressed on neuronal cells, whilst TRPC3 channels are present mainly on radial glial cells (Boisseau et al., 2009) Louhivuori and Jansson unpublished data). Thus it would seem that the effect of disrupting TRPC1 channels is directly affecting the neuronal cells. The mechanism by which disruption of TRPC1 function stimulates motility is unclear. However similar results have been obtained in GN11 cells. The data in these cells suggest that TRPC1 interferes with the function of other TRPC channels including TRPC3 and that lack of functional TRPC1 would promote the function of TRPC mediated processes. In this context it is interesting that BDNF and lack it TRPC1 shows similar properties like increase in motility without reduction in turning frequency or interaction with radial processes.

The migration of neural progenitor cells is promoted by neurotrophins like BDNF also in vivo(reviewed in (Dicou, 2009). This migratory response is inhibited by tyrosin kinase inhibitors such as K252a, inhibitors of phosphatidyl inositol-3-kinase (PI3K), and buffering of intracellular Ca^{2+} (Behar et al., 1997; Chiaramello et al., 2007; Polleux et al., 2002). The target for the action of BDNF seems to be a specific subpopulation (Ohmiya et al.) of progenitors (Alcantara et al., 2006; Polleux et al., 2002). As discussed above BDNF has been shown to activate TRPC3 channels in several studies and interference with the function of TRPC3 channels blocks chemotactic responses to BDNF. The coupling between activation of TrkB/protein kinases vs TRPC3 has not been resolved.

5.7 Role of voltage activated calcium channels in neuronal migration (IV)

Our work in Article III demonstrates that TRPC channels may play a central role in the interaction between radial glia cells and neuronal cells. As discussed above TRPC channels are nonselective cation channels with relatively low permeability to Ca^{2+}. They are thus expected to cause local depolarization due to influx of Na^{+} ions. As discussed above this could lead to Ca^{2+} elevations due to opening of voltage gated calcium channels with consequent influx of Ca^{2+}. Another possibility is that local depolarization and elevated intracellular Na^{+} would cause a reversal of Na^{+}/Ca^{2+} exchange towards Ca^{2+} influx as demonstrated in article II. The response to DHPG in radial glial cells was blocked by SN6, a selective inhibitor for the reverse mode of the Na^{+}/Ca^{2+} exchanger.

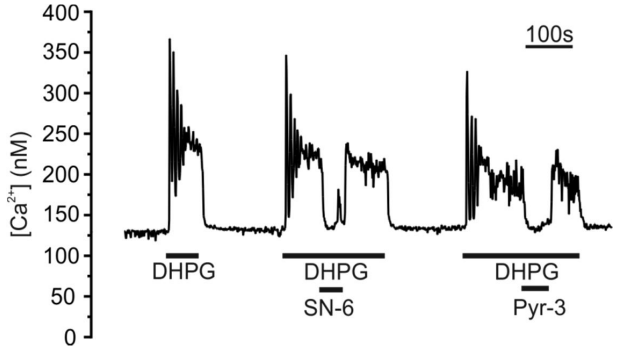

Figure 5: DHPG induced calcium response blocked by the NCX blocker SN-6.

We set out to investigate VGCC in embryonic NPCs in Article IV. We found mRNA expression of L-type VGCC Cav1.2 and Cav1.3 and T-type VGCC Cav3.1, Cav3.2 and Cav3.3 in the neurosphere derived migrating cells. When we stimulated embryonic NPCs with depolarizing concentrations of extracellular potassium during the very early stages of differentiation (1D and under), nearly all (<90%) of the cells gave a calcium response. Remarkably, HVA blockers were relatively ineffective except in the in the outer migration layers containing neurons (Article IV: Fig.4A). To our astonishment, the LVA channel blockers mibefradil and NNC effectively blocked the calcium rise (Article IV: Fig.4B). This response as well as immunostaining for T-type calcium channels was highest in the inner migration layers, containing radial glia, closer to the neurosphere. The calcium response to DHPG was blocked by NNC suggesting that T-type VGCC mediate the response via mGluR5.

Electrophysiological measurements demonstrated functional LVA channel expression (I-V ramp profiles, barium vs calcium ion selectivity, sensitivity to low concentrations of Ni^{2+}) (Article IV: Fig.3). The VGCC profile changed with the differentiation period, with HVA channel dominating the calcium response in later stages of differentiation when clear neuronal characteristics are present (i.e. correlating with the appearance of TTX sensitive voltage gated sodium channels: Article IV: Fig.6).

When neurospheres were grow in the presence of LVA channel blockers, unlike the subtle changes seen with TRPC channel disruption, there was a drastic effect on cell attachment, migratory capacity and neurite lengths of the NPCs (Article IV: Fig.9). Thus, this work provided one of the first evidences that LVA calcium channels are involved in the physiology of differentiating and migrating embryonic NPCs

Calcium influx via voltage gated calcium channels are also thought to play an important role in brain development (Komuro and Rakic, 1998; Spitzer, 1994). L-type voltage gated calcium channels have previously been reported to be functionally expressed in differentiated postnatal NPCs correlating with immunostained neuronal markers (D'Ascenzo et al., 2006). As well as a number of papers displaying a strong role for N-type VGCC in directing young postnatal purkinje cell migration (Rakic, 1971; Rakic, 1972; Rakic, 1988). Nonetheless, notwithstanding the clear role of VGCC in neural development (reviewed in (Rakic, 2006), the expression and function of these channels during embryonic neurogenesis are less well investigated.

6. Conclusion

These studies have provided valuable new information on the central role TRP channels play as integrative sensors of complex chemical signals in differentiating neuronal cells. One of the principal finding is the concept that TRPC channels mediate primarily the influx of sodium which would then lead to the influx of calcium through other pathways. One such pathway would be via the sodium calcium exchanger; by increasing the sodium concentration close to NCX it would drive the NCX to operate in the reverse mode (calcium influx). This potential protein complex (receptor/channel/exchanger) might behave as a local module whereby signals from the cell's environment are integrated to minute changes in the microdomains surrounding this complex thus avoiding global changes in ion concentrations. Another pathway would involve the gating of low voltage activated calcium channels. The depolarizing effect of cations entering via TRPC may provide the small depolarization steps needed to activate LVA channels.

Secondly this thesis work provided one of the first insights into the physiological role of LVA channels in early neural cell differentiation and migration. Disrupting LVA activity had a drastic effect on neural cell migration bringing it to a near complete halt and further more disrupted the extension of neurite processes. The larger implications of this finding other than the cellular physiological insight, relates for example to the possible adverse effects medications targeting these channels (such as those used to treat epilepsy) in the CNS may have on the neural stem cell niches present in adults. Even though the role of adult neurogenesis is still unclear, it is well accepted that injury enhances proliferation of quiescent stem and progenitor cells in specialized niches within the brain, and may also be involved in the cognitive decline frequently observed with chronic epilepsy (Snyder et al., 2011). Could it be that drugs designed to help increase the quality of life of these patients be adding to the adverse effect observed via their disruption of neural stem cell function? More studies are required to address these questions.

Thirdly metabotropic glutamate receptors in association with TRPC channels play an integral role in the interaction between glia and neuronal cells. Disrupting this signaling complex led to disturbances in the migratory behavior of neuronal cells and reduced the contact based interaction between neurons and glial cells in vitro. However how do these data support the living animal? TRPC3 knockout mouse display a rather mild phenotype. In the cerebellum, where granule cell migration along radial glial fibers is a paradigm of glia-mediated neuronal guidance, the neuronal organization of molecular, Purkinje and granular layer appear perfectly preserved. Firstly, impaired laminar brain organization is not a prerequisite for deficits in cognitive function. Many syndromes of

mental retardation do not show any macroscopic morphological changes. Secondly, a strong case for the effect of mGluR5 knockout studies indicates that alterations in cell segregation takes place in specific areas of cortical layer IV as well as in dendritic spine morphology and density (Chen et al., 2012; Wijetunge et al., 2008). These studies underlie the potential role in which mGluR5 receptors take part in influencing synaptic function in the brain. Interestingly, the changes seen varied with age. This would suggest that compensatory mechanisms are taking place to mask the initial genetic insult. In this context our results investigate the embryonic stage of development where by the genetic insult is near or at its zenith. Our finding that the interaction between glial cells and neuronal progenitor cells is impaired leading to changes in cell motility when TRPC3/mGluR5 channels are blocked with alterations in the calcium signaling brought about via mGluR5 may perpetuate itself in synaptic function. Impairing TRPC/mGluR5 function reduced contact based signaling between the progenitor cells and the radial glial network. Thirdly, many of the studies done on knockout animals have not delved deep into the microscopic anomalies that may be present (for example hardly any studies have been done investigating the glial cell populations).

Thus the studies in this thesis highlight the important role Ca^{2+} influx pathways have in mediating a significant role in the neural development and maturation of neuronal cells. It is the hope of the author that these new and valuable pieces of information that have come to light will help future research unlock the secrets still hidden.

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8. Appendix